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# Pancreatic differentiation of *Pdx1-GFP* reporter mouse induced pluripotent stem cells

Angelo Porciuncula,[a, b, 1, 2] Anujith Kumar,[c, 2, 3] Saray Rodriguez,[a, b] Maher Atari,[d] Miriam Araña,[a] Franz Martin,[e] Bernat Soria,[e] Felipe Prosper,[a, b] Catherine Verfaillie,[c, f] Miguel Barajas [a, b, *, 4]

[a] *Centro de Investigación Médica Aplicada (CIMA), University of Navarra, Avda. Pio XII 55, Pamplona 31008, Spain*
[b] *Hematology and Cell Therapy Area, Clínica Universidad de Navarra, Avda. Pio XII 36, 31008 Pamplona, Spain*
[c] *Stem Cell Institute, KU Leuven, Leuven 3000, Belgium*
[d] *Regenerative Medicine Research Institute, UIC Barcelona, Barcelona, Spain*
[e] *CABIMER, Andalusian Center for Molecular Biology and Regenerative Medicine, Seville 41092, Spain*
[f] *Department of Development and Regeneration, Stem Cell Biology and Embryology, KU Leuven, Leuven 3000, Belgium*

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ABSTRACT

Efficient induction of defined lineages in pluripotent stem cells constitutes the determinant step for the generation of therapeutically relevant replacement cells to potentially treat a wide range of diseases, including diabetes. Pancreatic differentiation has remained an important challenge in large part because of the need to differentiate uncommitted pluripotent stem cells into highly specialized hormone-secreting cells, which has been shown to require a developmentally informed step-by-step induction procedure. Here, in the framework of using induced pluripotent stem cells (iPSCs) to generate pancreatic cells for pancreatic diseases, we have generated and characterized iPSCs from *Pdx1-GFP* transgenic mice. The use of a GFP reporter knocked into the endogenous *Pdx1* promoter allowed us to monitor pancreatic induction based on the expression of *Pdx1*, a pancreatic master transcription factor, and to isolate a pure Pdx1-GFP$^+$ population for downstream applications. Differentiated cultures timely expressed markers specific to each stage and end-stage progenies acquired a rather immature beta-cell phenotype, characterized by polyhormonal expression even among cells highly expressing the Pdx1-GFP reporter. Our findings highlight the utility of employing a fluorescent protein reporter under the control of a master developmental gene in order to devise novel differentiation protocols for relevant cell types for degenerative diseases such as pancreatic beta cells for diabetes.



## 1. Introduction

Induced pluripotent stem cells (iPSCs) offer a superior advantage over embryonic stem cells (ESCs) in that they are capable of generating immune-compatible replacement cells and patient-specific disease models for a wide range of diseases (Robinton and Daley, 2012). The rate-limiting step, however, for any application to emerge from both iPSCs and ESCs is their efficient and correct differentiation towards relevant cell types, such as pancreatic beta cells for the treatment and study of diabetes mellitus (Soria et al., 2015).

Most differentiation protocols towards the pancreas have been designed based on principles of development (Spence and Wells, 2007). With the administration of cytokines, growth factors, and small molecules, pluripotent stem cells have been coaxed *in vitro* to differentiate following a sequence of steps leading to the pancreatic beta cell. After passing through a transient primitive streak-like mesendoderm stage (Tada et al., 2005), pluripotent stem cells differentiate into a Sox17$^+$ and FoxA2$^+$ population known as the definitive endoderm (DE), the germ layer responsible for forming major organs like the liver, pancreas, lung, gut, and intestine (Wells and Melton, 1999). This step is mediated by exposure to high concentrations of TGF-beta family member Activin-A, which has been shown to mimic the functions of co-member Nodal in endoderm induction (Kubo et al., 2004).

DE cells, upon the repression of sonic hedgehog (Shh) signaling (Hebrok et al., 1998) activate the pancreatic program centered on the homeobox gene *Pdx1* (Jonsson et al., 1994) which marks all pancreatic derivatives. Pdx1$^+$ epithelia have been shown to be capable of proliferation as progenitors (Zhou et al., 2007), and, during a narrow timeframe during embryogenesis, are decisive in determining the final organ size of the pancreas (Stanger et al., 2007). The suppression of Notch (Apelqvist et al., 1999) later promotes the specification of the endocrine lineage. The final step is the maturation of endocrine progenitors into insulin-producing beta cells.

Numerous reports have described differentiation of pluripotent stem cells *in vitro* towards the aforementioned developmental stages: definitive endoderm (Kubo et al., 2004; D'Amour et al., 2005; Yasunaga et al., 2005), pancreatic endoderm (Kroon et al., 2008; Ku et al., 2004; Micallef et al., 2005), and insulin-producing cells (D'Amour et al., 2006; Pagliuca et al., 2014; Rezania et al., 2014; Russ et al., 2015). Despite these considerable advances, many unresolved issues remain like the heterogeneity of differentiated cultures,

* Corresponding author at: Biochemistry Department, Faculty of Health Science, Public University of Navarre (UPNA), Avda. Barañain, s/n | E-31008 - Pamplona | Spain.
Email address: miguel.barajas@unavarra.es (M. Barajas)

[1] Present address: Department of Biochemistry & Molecular Pharmacology, New York University School of Medicine.

[2] These authors contributed equally to this work.

[3] Present address: School of Regenerative Medicine, Manipal University, Bangalore-560065, India.

[4] Present address: Biochemistry Area. Health Science Department. Faculty of Health Science. Public University of Navarre (UPNA).

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the irreproducibility of published protocols due to clonal variability and intrinsic differences in laboratory conditions, and the inefficient production of mature differentiated cell types. In this report, we generated reporter iPSCs from transgenic *Pdx1-GFP* knock-in mice and differentiated them towards pancreatic cells using a novel protocol. At the end of the differentiation, we sorted Pdx1-GFP$^+$ cells and detected expression of multiple hormone markers, showing induction of endocrine cells, albeit with an immature phenotype. These findings contribute to the large body of evidence of the pancreatic differentiation potential of iPSCs and may aid towards the design of a more robust pancreatic differentiation protocol for a wide range of pluripotent cell lines.

## 2. Materials and methods

### *2.1. Generation of iPSCs*

iPSCs were generated from *Pdx1-GFP* knock-in reporter mouse (Gu et al., 2002)-derived mouse embryonic fibroblasts (MEFs) by the retroviral transduction of Oct4, Sox2, and Klf4 (Addgene) as published with minor modifications (see Supplementary Methods) (Takahashi and Yamanaka, 2006). iPSC colonies were picked up on day 20 post-infection and expanded as stable clones under standard ESC culture conditions. For details of iPSC generation and characterization, see Supplementary Methods.

### *2.2. Cells*

Mouse iPSCs and ESCs were maintained on mitomycin-C (Sigma)-inactivated MEFs in ESC medium containing DMEM with 4.5 g/l glucose (Gibco), 15% fetal bovine serum (Hyclone), $10^3$ U/l leukemia inhibitory factor (Millipore), 1× MEM non-essential amino acids (Gibco), 2 mM L-glutamine (Lonza), 0.1 mM beta-mercaptoethanol (Gibco), and 1× penicillin/streptomycin (Gibco). Cells were trypsinized with 0.05% trypsin/EDTA (Gibco) and passaged at 1:6 or higher every 2–3 days.

### *2.3. Pancreatic differentiation of iPSCs*

iPSCs were previously grown for one passage on 0.1% gelatin (Millipore) to dilute MEF feeders. After two days, iPSCs were seeded on 12-well plates coated with 2% growth factor-reduced Matrigel (BD) at 5000 cells/cm$^2$. Cells were maintained continuously in basal differentiation medium containing DMEM with 1 g/l glucose (Gibco), 40% MCDB 201 (Sigma), 100 μM L-ascorbic acid (Sigma), 0.25× linoleic acid/BSA (Sigma), 0.25× ITS (Sigma), 50 μM beta-mercaptoethanol (Gibco), and 1× penicillin/streptomycin (Gibco). Cells were treated with the following cytokines and small molecules as follows with medium change every other day (Fig. 2): days 1–3: 100 ng/ml Activin-A (R&D) and 50 ng/ml Wnt3a (R&D) in 2% FBS; days 3–6: 100 ng/ml Activin-A only in 1% FBS; days 6–8: 2.5 μg/ml monoclonal Shh antibody (R&D), 2 μM all-trans RA (Sigma), 50 ng/ml KGF (R&D), and 100 ng/ml noggin (R&D) in 0.5% FBS; days 8–10: same previous medium was used but without noggin; days 10–16: 50 ng/ml KGF and 50 ng/ml heparan sulfate (Sigma) without serum; and days 16–22: 50 ng/ml betacellulin (R&D), 10 nM exendin-4 (Sigma), 10 mM nicotinamide (Sigma), and 50 ng/ml GDF11 (R&D) without serum.

### *2.4. qpcr analysis*

Total RNA was isolated from cultured cells using the RNAEasy Micro Kit (Qiagen). Reverse transcription was performed using the Superscript II RT kit (Invitrogen) and quantitative PCR (qPCR) using the SYBR Green Master Mix (Invitrogen). qPCR was carried out using the ECycler1000 thermocycler (Eppendorf). qPCR data were expressed in Delta-Ct values, i.e., Ct values of gene of interest – Ct values of housekeeping gene *Gapdh* (Fig. 1c and d), in relative expression, i.e., fold-change values with respect to day 0 (Figs. 3a, 4a and c), or in Log fold-change, i.e., logarithm of fold-change compared to day 0 (Fig. 5c).

### *2.5. Immunofluorescence*

Cells were fixed in 10% neutral buffered formalin for 30 min, washed with PBS, and then blocked and permeabilized with 5% horse serum, 3% bovine serum albumin (BSA) (Sigma), and 0.3% Triton X-100 (Sigma) in PBS for another 30 min. Primary antibodies were added in their appropriate dilutions in 1% serum (from the same species as the source of their corresponding secondary antibodies), 3% BSA, and 0.1% Triton X-100 in PBS overnight at 4 °C. The next day, secondary antibodies were added in the same antibody diluent at 1:500 and incubated for 1 h and 30 min at room temperature in dark. Nuclei were stained with Hoechst (4 μg/ml) (Sigma) in simultaneous incubation with the secondary antibody. Washes with 1× PBS were performed in between steps except before incubation with primary antibody.

For double immunostaining, procedure from the blocking and permeabilization step and onwards was repeated after the first secondary antibody incubation on the second day. Cells were incubated with the second set of primary antibodies overnight at 4 °C. Co-staining with the second set of secondary antibodies and Hoechst was done on the third day. Cells were viewed under an inverted fluorescence microscope (Zeiss).

### *2.6. Cell sorting*

Day 22 cultures were dissociated with treatment of dispase (BD) for 30 min followed by 0.25% trypsin/EDTA (Gibco) for 10 min at 37 °C until they could be mechanically disaggregated into single cells with a micropipette. Cells were resuspended in flow cytometry buffer (3% FBS, 25 mM HEPES, 5 mM EDTA in PBS) and maintained at 4 °C ready for cell sorting. Prior to sorting, cell viability was assessed by Trypan blue dye exclusion. Sorting gates were set for GFP$^+$ population. GFP$^+$ cells were analyzed and separated with the FACSAria II cytometer (BD). Debris, doublets, and dead cells were all excluded by forward scatter, side scatter, and TOPRO gating. Gating was implemented based on staining profiles of undifferentiated iPSCs as negative control. A post-sorting analysis was performed for both GFP$^+$ and GFP$^-$ cells to ensure purity of sorted populations. Sorted cells were collected in lysis buffer (Qiagen) in preparation for RNA analysis.

### *2.7. Statistics*

Statistical analysis of qPCR data was performed using 1-way ANOVA followed by a Bonferroni's multiple comparison test on mean-centered values using the GraphPad Prism Version 6 software. Mean-centered values were calculated as previously described (Willems et al., 2008) in order to normalize highly variable data

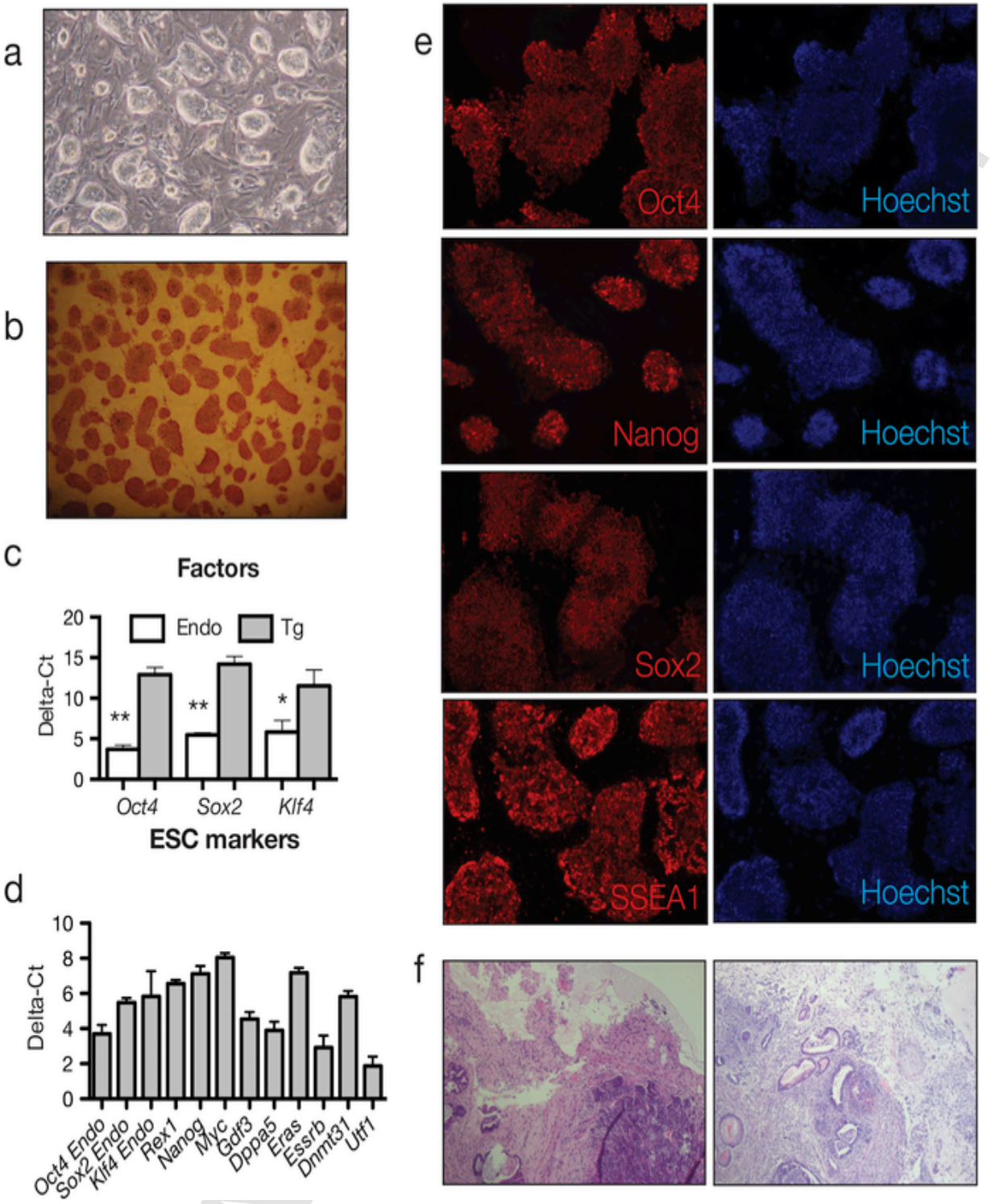


**Fig. 1.** Characterization of *Pdx1-GFP* iPSC clones. (a) Brightfield micrograph of representative iPSC clone resembling ESCs in morphology. (b) Staining for alkaline phosphatase. (c) Retroviral silencing of exogenous factors detected by qPCR using primers that specifically detect endogenous (Endo) and exogenous or transgenic (Tg) transcripts; data expressed in Delta-Ct or change in cycle thresholds (i.e., $Ct_{Gapdh}$–$Ct_{Gene}$) with respect to housekeeping gene *Gapdh* ($n$=2 clones in triplicates). (d) Induction of ESC markers detected by qPCR; data expressed in Delta-Ct ($n$=2 clones in triplicates). Oct4, Sox2, and Klf4 transcripts were detected from endogenous (Endo) loci. (e) Immunostaining for Oct4, Nanog, Sox2, and SSEA1 in representative iPSC clone. Nuclei stained with Hoechst. (f) Teratoma formation of representative iPSC clone showing tissues of the three germ layers.

across biological replicates. Statistical significance is indicated as follows: * $p<0.05$; ** $p<0.01$; *** $p<0.001$.

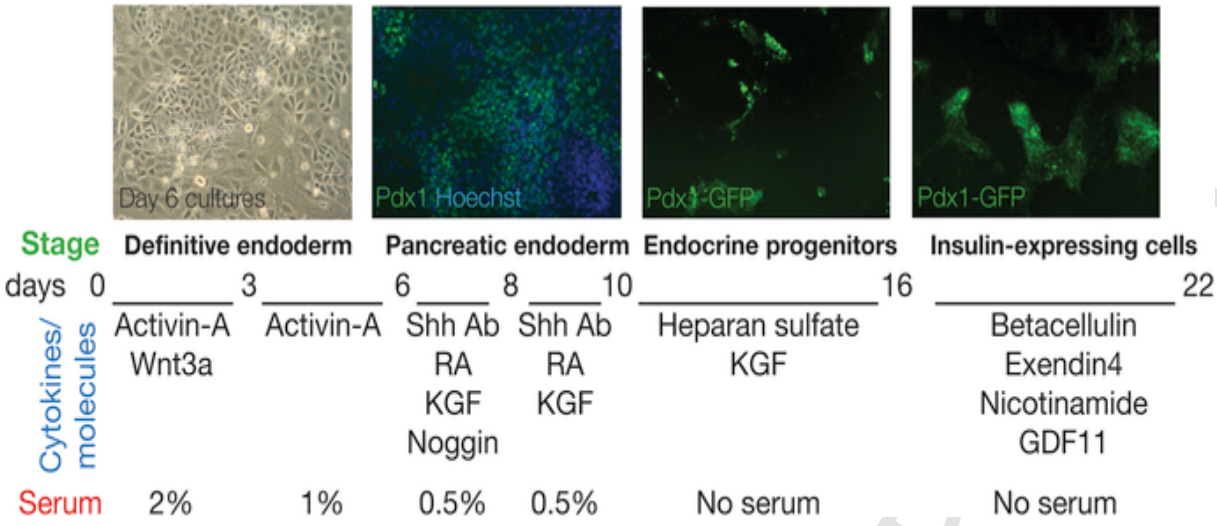


**Fig. 2.** Pancreatic differentiation protocol. Schematic diagram of differentiation protocol in four stages: definitive endoderm (days 0–6), pancreatic endoderm (days 7–10), endocrine progenitors (days 11–16), and insulin-expressing cells (days 17–22). Micrographs show sample differentiation cultures by stage: flat, polygonal endoderm sheets by day 6, endogenous Pdx1 activation by day 10, Pdx1-GFP visualization by day 16, which increases significantly in number by day 22. Diagram also shows the cytokines and small molecules used in the protocol, and the decreasing serum concentrations employed across the four stages.

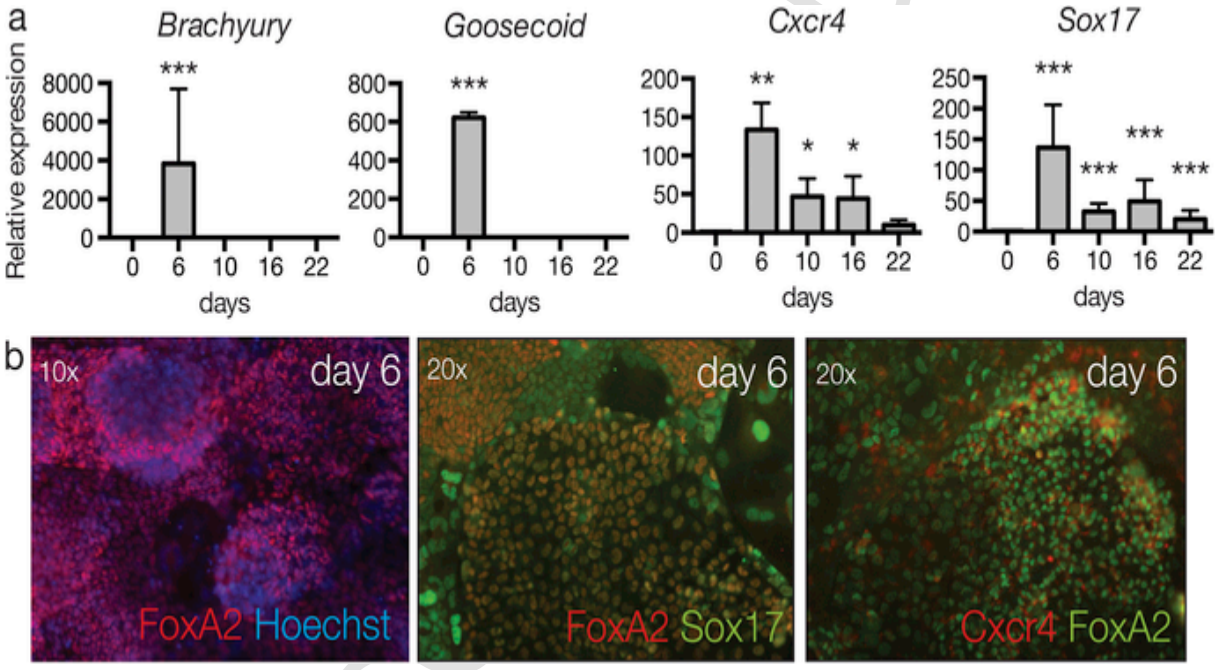


**Fig. 3.** Definitive endoderm (DE) induction in iPSCs. (a) Induction of primitive streak markers *Brachyury* and *Goosecoid* and DE markers *Cxcr4 and Sox17*. *n*=2 experiments for one clone. (b) Day 6 double immunostaining for FoxA2 and Hoechst (10× objective magnification), FoxA2 and Sox17 (20×), and Cxcr4 and FoxA2 (20×).

## 3. Results and discussion

### *3.1. Generation and characterization of iPSCs from Pdx1-GFP reporter mice*

Following a standard protocol (Takahashi et al., 2007), MEFs derived from transgenic mice carrying a *GFP* reporter under the control of the endogenous *Pdx1* promoter (Gu et al., 2002) were reprogrammed into iPSCs using the three reprogramming factors *Oct4, Sox2, and Klf4* (Fig. 1a). Reprogramming factor Myc was omitted from the reprogramming cocktail in order to achieve a more uniform and complete reprogramming and to minimize *in vivo* tumorigenicity due to the potential reactivation of retroviral Myc (Nakagawa et al., 2008). While a lot more colonies were observed among Myc-infected cultures, the probability of isolating partially or incompletely reprogrammed clones, characterized by the absence of transgenic silencing (Maherali and Hochedlinger, 2008), was higher in these cultures than in Myc-free infected cells (data not shown). Thus, to avoid the possibility of isolating partially or incompletely reprogrammed clones, all work presented in this study was performed on Myc-free iPSCs.

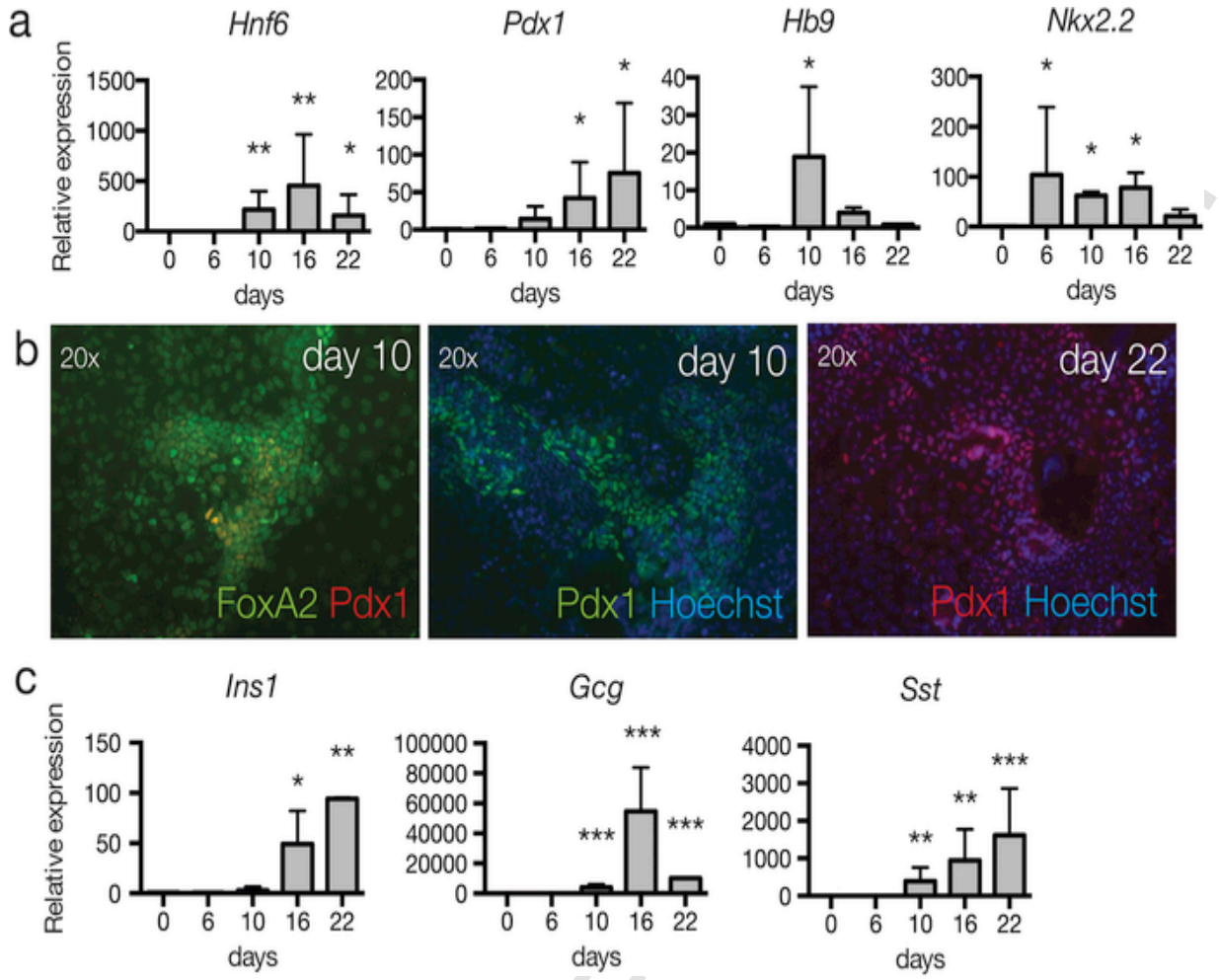


**Fig. 4.** Pancreatic induction in iPSCs. (a) Activation of pancreatic endoderm markers Hnf6, Pdx1, Hb9, and Nkx2.2 detected by qPCR. *n*=2 experiments for one clone. (b) Double immunostaining for Pdx1 and Hoechst (day 10), FoxA2 and Pdx1 (day 10), and Pdx1 and Hoechst (day 22). All images were taken at 20x objective magnification. (c) Induction of hormone markers *Ins1* (*Insulin1*), *Gcg* (*Glucagon*), and *Sst* (*Somatostatin*) measured by qPCR. *n*=2 experiments for one clone.

Established iPSC clones expressed standard ESC markers such as alkaline phosphatase (Fig. 1b), the endogenous reprogramming factors (Fig. 1c and e), along with the rest of the pluripotency genes such as *Rex1*, *Nanog*, and *Gdf3*, among others (Fig. 1d). All clones tested were also capable of generating teratomas containing tissues representative of the three embryonic germ layers when subcutaneously injected into immunocompromised mice (Fig. 1f). These data show that at the level of morphology, growth properties, and expression of pluripotency markers, iPSCs were highly similar to ESCs.

### *3.2. Sequential addition of developmentally relevant cytokines leads to pancreatic induction*

To standardize the protocol, we tested numerous parameters known to influence pancreatic differentiation of pluripotent cells *in vitro*: seeding density, culture substrates, serum concentration, and the different cytokines, growth factors, and small molecules involved in driving pancreatic specification *in vivo*.

After one passage of iPSCs on gelatin in the absence of feeders, iPSCs were cultured as a monolayer at a low seeding density (5000 cells/cm$^2$) on Matrigel, a substrate previously used to differentiate towards DE (Kubo et al., 2004; Yasunaga et al., 2005). Cells were continuously cultured for 22 days in the presence of various cytokines and small molecules (Fig. 2), empirically chosen on the basis of: (a) induction of a panel of stage-specific developmental markers, and (b) induction of the *Pdx1-GFP* locus as monitored by GFP fluorescence.

During the first six days (days 1–6), in the presence of high concentration of Activin-A (Kubo et al., 2004; Yasunaga et al., 2005), Wnt3a (D'Amour et al., 2006), and in reduced serum conditions (Tada et al., 2005), cells proliferated and grew flat epithelial patches (Suppl. Fig. 1) that resembled endoderm cells as previously reported (Tada et al., 2005). These cells expressed key DE markers FoxA2, Sox17, and Cxcr4 in mRNA and protein (Fig. 3a and b). Primitive streak markers, *Brachyury* and *Goosecoid*, sharply peaked on or before day 6, but went undetected immediately after (Fig. 3a), suggesting that cells had passed through an intermediate mesendoderm stage (Tada et al., 2005).

For the next four days (days 6–10), cells were exposed to signaling molecules that drive pancreatic specification (Fig. 2): (a) monoclonal antibody against Shh (Hebrok et al., 1998), a less toxic substitute to the more routinely used KAAD-cyclopamine; (b) all-*trans* retinoic acid (RA), inducer of dorsal pancreatic endoderm (Martín et al., 2005); (c) keratinocyte growth factor (KGF), a factor that promotes proliferation of pancreatic epithelia (Ye et al., 2005); and (d) Noggin, a BMP inhibitor to exclude the hepatic fate (Mfopou et al.,

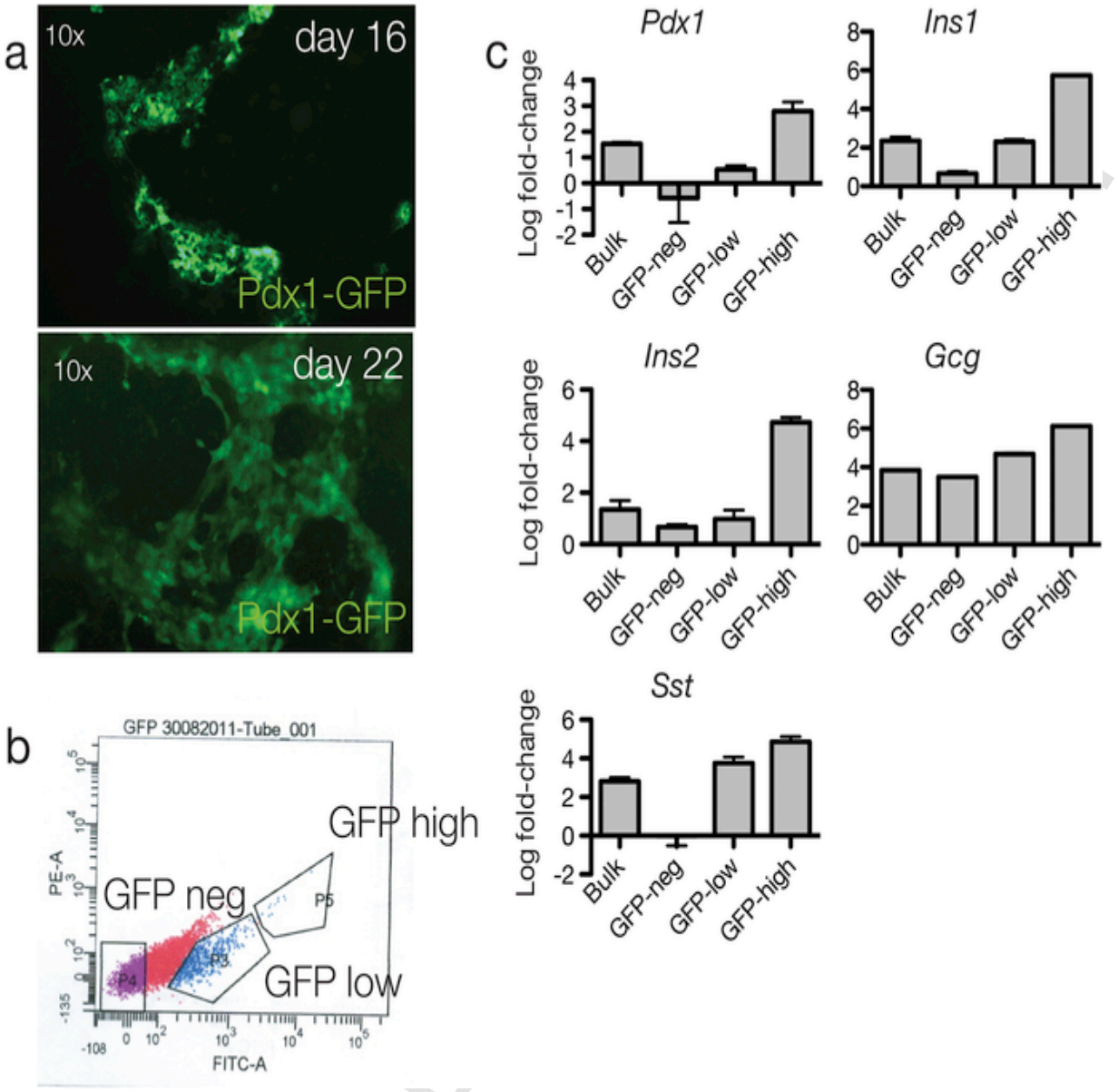


**Fig. 5.** Sorted GFP$^+$ cells express multiple hormone markers. (a) Fluorescence micrographs of Pdx1-GFP$^+$ clusters on day 16 and 22 (both at 10× objective magnification). (b) Representative flow cytometry analysis showing sorted GFP-negative (GFP neg), GFP low, and GFP high populations. (c) Detection of transcripts of *Pdx1* and key hormone markers *Ins1* (*Insulin1*), *Ins2* (*Insulin2*), *Gcg* (*Glucagon*), and *Sst* (*Somatostatin*) in bulk (or unsorted) day 22 cultures, and sorted GFP-neg, GFP-low, and GFP-high populations. *n*=2 experiments for one clone.

2010). *Pdx1* transcripts (Fig. 4a) and proteins (Fig. 4c) were first detected on day 10 and steadily increased towards the end of the protocol (Fig. 4a and b). *Hnf6*, an upstream activator of *Pdx1* (Jacquemin et al., 2003), was activated on day 10 and maintained high levels throughout the culture (Fig. 4a). Other markers of pancreatic epithelial precursors like *Hb9* and *Nkx2.2* were likewise induced on day 10 (Fig. 4a), indicating pancreatic endoderm induction.

To expand these Pdx1$^+$ cells, KGF treatment was extended for six more days (days 10–16) along with the addition of heparan sulfate (Fig. 2), a proteoglycan expressed in pancreatic epithelial basement membrane and mesenchyme that maintains the pancreatic progenitor pool and delays its differentiation (Zertal-Zidani et al., 2007), and, upon the binding of KGF, promotes epithelial branching morphogenesis (Makarenkova et al., 2009). On day 16, *Pdx1* transcripts increased by four-fold (Fig. 4a), corroborated by robust Pdx1 immunostaining in later day 22 cultures (Fig. 4b). Co-immunostaining for FoxA2 and Pdx1 confirms that Pdx1$^+$ cells arise from previously expressing FoxA2$^+$ cells (Fig. 4b).

The final phase (days 16–22) consisted in treating cells with factors that favor beta-cell maturation and expansion in vitro (Fig. 2): betacellulin (Huotari et al., 2002), exendin-4 (List and Habener, 2004), nicotinamide (Otonkoski et al., 1993), and GDF11 (Harmon et al., 2004). Day 22 cultures expressed islet hormone markers indicating the cells had undergone pancreatic endocrine commitment. Transcripts of *Glucagon* (*Gcg*), expressed by the α-cell, and *Somatostatin* (*Sst*), by the δ-cell, were detected as early as in day 10 cultures and were continued to be expressed by more than a 10,000- and 1000-fold, respectively, whereas *Insulin1* (*Ins1*), proper of the beta-cell, was induced later at day 16 at levels similar to those of *Pdx1* (Fig.

4c). By contrast, the other insulin isoform, *Insulin2* (*Ins2*), equally functional in rodents as *Ins1* (Giddings and Carnaghi, 1988) was not detected in our cultures (data not shown). These data suggest that endocrine commitment of iPSCs *in vitro* in part resembles that of embryonic development in that *Gcg*-expressing cells arise earlier followed by *Ins*-expressing ones, but differs in that *Sst*-expressing cells were likewise induced earlier.

An important consideration in differentiation *in vitro* is the spontaneous induction of divergent lineages that could be occurring at the expense of pancreatic differentiation. First, residual expression of pluripotency marker *Oct4* was considerably reduced especially after day 6 (Suppl. Fig. 2a); however, it was still detected in end-stage cultures, albeit in few clusters of cells (Suppl. Fig. 2b). The expression of markers of primitive endoderm *Sox7* and of visceral endoderm *Afp* (Suppl. Fig. 2a) suggests induction of extraembryonic cells. However, *Sox7* is also known to mark mesoderm (Gandillet et al., 2009) and *Afp* both embryonic mesoderm (Dziadek and Adamson, 1978) and early hepatoblasts (Gualdi et al., 1996), suggesting that their induction may not be completely due to the extraembryonic lineage. Besides, the highest *Sox7* induction occurred much later at day 16 (Suppl. Fig. 2a), arguing in part its expression in mesoderm-derived cells, which in the context of pancreatic differentiation could possibly be participating in crucial epithelial-mesenchymal interactions (Jacquemin et al., 2006). On the other hand, the other more mature liver marker, *Albumin* (*Alb*), was only mildly induced towards the end of the protocol compared to *Afp* (Suppl. Fig. 2a), which does not rule out completely hepatic induction. Lastly, neuroectoderm marker *Sox1* was not detected at any stage of the protocol (data not shown). Thus, more than having yielded liver, cells might have spontaneously differentiated into non-pancreatic lineages that mainly include the mesoderm and the primitive endoderm.

### *3.3. Pdx1-GFP$^+$ cells arise in culture on day 16*

The main advantage of using a *Pdx1-GFP* knock-in reporter line is the ability to monitor induction by visualizing GFP fluorescence. Despite induction of Pdx1 at both mRNA and protein levels earlier on day 10 (Fig. 4a and b), cells did not appear Pdx1-GFP$^+$ till around day 16 (Fig. 5a), suggesting that KGF treatment was necessary to expand nascent Pdx1$^+$ cells in culture confirming published data on pancreatic tissue explants (Ye et al., 2005). Pdx1-GFP$^+$ cells formed in clusters that proliferated towards the end of the protocol (Fig. 5a). The apparent discrepancy in the timing of detection between induction of the *Pdx1-GFP* locus and endogenous Pdx1 may be due to the weak intensity of the reporter at earlier timepoints when there is assumingly less Pdx1 expression, as immunostaining of day 10 cultures detected GFP$^+$ cells (Suppl. Fig. 3). Finally, GFP$^+$ cells were also verified by double immunostaining to be simultaneously co-expressing Pdx1 (Suppl. Fig. 3) confirming non-leaky expression of the reporter transgene.

### *3.4. Pdx1-GFP$^+$ cells have an immature endocrine phenotype*

The Pdx1-GFP knock-in reporter system allows the isolation of a pure population of differentiated Pdx1$^+$ pancreatic cells. From day 22 cultures (Fig. 5a), by cell sorting, both GFP$^{high}$ and GFP$^{low}$ populations were isolated (Fig. 5b). Both GFP$^{high}$ and GFP$^{low}$ cells expressed high levels of *Ins1*, Gcg, and *Sst*, with higher induction in the GFP$^{high}$ population as expected, while no induction was observed in the GFP$^-$ population (Fig. 5c). *Ins2*, undetected in bulk cultures, was detected only in the GFP$^{high}$ population, suggesting that high levels of *Pdx1* are required for this marker (Fig. 5c). Interestingly, the GFP$^-$ population, while negative for *Ins1*, *Ins2*, and *Sst*, expressed comparatively high levels of *Gcg*, suggesting the induction of either a significant number of α-cells or of cells that had undergone primary transition as in development (Gittes, 2009). The co-expression of all three hormones in the GFP$^+$ population indicates the immature phenotype of the pancreatic endocrine cells generated.

Here, we have described a novel pancreatic differentiation protocol using a fluorescent reporter indicative of expression of master pancreatic transcription factor *Pdx1*. As in other *in vitro* protocols, differentiated progenies yielded an immature pancreatic endocrine phenotype marked by polyhormonal expression (Hrvatin et al., 2014). Further maturation could be enhanced by *in vivo* transplantation (Kroon et al., 2008; Rezania et al., 2012) or, theoretically, by adding beta-cell maturation and expansion factors as observed in tissue explant experiments (List and Habener, 2004).

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## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.diff.2016.04.005.

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